

EXHIBIT 19

Primary, Syncytium-Inducing Human Immunodeficiency Virus Type 1 Isolates Are Dual-Tropic and Most Can Use Either Lestr or CCR5 as Coreceptors for Virus Entry

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A panel of primary syncytium-inducing (SI) human immunodeficiency virus type 1 isolates that infected several CD4⁺ T-cell lines, including MT-2 and C8166, were tested for infection of blood-derived macrophages. Infectivity titers for C8166 cells and macrophages demonstrated that primary SI strains infected macrophages much more efficiently than T-cell line-adapted HIV-1 strains such as LAI and RF. These primary SI strains were therefore dual-tropic. Nine biological clones of two SI strains, prepared by limiting dilution, had macrophage/C8166 infectivity ratios similar to those of their parental viruses, indicating that the dual-tropic phenotype was not due to a mixture of non-SI/macrophage-tropic and SI/T-cell tropic viruses. We tested whether the primary SI strains used either Lestr (fusin) or CCR5 as coreceptors. Infection of cat CCC/CD4 cells transiently expressing Lestr supported infection by T-cell line-adapted strains including LAI, whereas CCC/CD4 cells expressing CCR5 were sensitive to primary non-SI strains as well as to the molecularly cloned strains SF-162 and JR-CSF. Several primary SI strains, as well as the molecularly cloned dual-tropic viruses 89.6 and GUN-1, infected both Lestr⁺ and CCR5⁺ CCC/CD4 cells. Thus, these viruses can choose between Lestr and CCR5 for entry into cells. Interestingly, some dual-tropic primary SI strains that infected Lestr⁺ cells failed to infect CCR5⁺ cells, suggesting that these viruses may use an alternative coreceptor for infection of macrophages. Alternatively, CCR5 may be processed or presented differently on cat cells so that entry of some primary SI strains but not others is affected.

Human immunodeficiency virus type 1 (HIV-1) and HIV-2 strains enter cells by interacting with both CD4 and a coreceptor at the cell surface. Coreceptors are determinants of virus tropism so that viruses with distinct tropisms use different coreceptors during entry. Recently Lestr (also called fusin) (18, 20, 21, 24, 31), a member of a large family of G-protein-coupled receptors with seven-transmembrane domains including the more closely related CC and CXCR chemokine receptors (4, 13, 30, 34-36), has been shown to be a coreceptor for T-cell line-adapted HIV-1 strains (19). Several studies have since shown that CCR5 acts as a coreceptor for macrophage-tropic non-syncytium-inducing (NSI) strains (1, 15, 17, 42), although at least some NSI strains can use either CCR3 or CCR5 (6). Furthermore, we and others have shown that individual virus strains can be promiscuous, using one of several compatible coreceptors (3, 16).

The majority of HIV-1 isolates from infected asymptomatic patients display an NSI phenotype and generally fail to infect most established CD4⁺ T-cell lines (2). Primary HIV-1 strains that can infect and induce syncytia in T-cell lines can, however, be isolated from many patients with AIDS (14, 39). Little is known about how this property influences pathogenesis, transmission, or tropism in vivo. Individuals that yield such strains, however, are more likely to suffer a faster disease progression (14). In vitro, HIV-1 isolates passaged in and adapted to hu-

man CD4⁺ T-cell lines are likely to evolve into syncytium-inducing (SI) strains even if they are NSI to begin with. Such adapted viruses usually infect primary macrophages inefficiently, although some that are more proficient for macrophage infection have been termed dual-tropic (12, 26, 37). It is less clear whether primary strains that can infect T-cell lines in vitro have concomitantly evolved a reduced tropism for macrophages. Valentin et al., however, showed that all primary isolates that they tested, as well as T-cell line-adapted LAI, infected macrophages at least to some extent (40). Here, we show that a panel of SI primary isolates infect primary human macrophages cultures more efficiently than T-cell line-adapted HIV-1 strains. Furthermore, several dual-tropic SI strains were able to use either Lestr or CCR5 as coreceptors, in contrast to T-cell line-adapted strains, which infected Lestr⁺ cells but not CCR5⁺ cells.

MATERIALS AND METHODS

Cells. Macrophages were prepared from blood monocytes by plastic adherence as previously described (37). The purity of macrophage preparations was tested at the time of infection. Over 99% of cells in macrophage preparations stained positive with an anti-CD14 monoclonal antibody (Sigma). Peripheral blood mononuclear cells (PBMCs) were stimulated for 2 days with phytohemagglutinin (0.5 µg/ml) and then cultured in medium with interleukin-2 (20 U/ml). CCC S+L- cells stably expressing recombinant human CD4 have been described previously (7, 25). CD4⁺ human T-cell lines used were MT-2 cells (28), which are indicator cells for SI strains (22), and C8166 cells (9), which are used to propagate and titrate T-cell line-adapted HIV-1 strains.

Viruses. LAI (41) and RF (33) are T-cell line-adapted HIV-1 strains. GUN-1 is also T-cell line adapted but dual-tropic (26, 37). 89.6 is an HIV-1 strain that infects macrophages but can infect and induce syncytia in certain CD4⁺ T-cell lines and is therefore also dual-tropic (12). SF-162 is an NSI/macrophage-tropic strain of HIV-1 (5), while JR-CSF is also NSI yet infects macrophages less efficiently

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TABLE 1. Infection of the C8166 T-cell line and primary cultures of PBMCs or macrophages by primary HIV-1 isolates

Virus strain	SI status	Infectivity titer (TCID ₅₀ /ml)			Ratio, macrophage infectivity/C8166 cell infectivity
		PBMCs	Macrophages	C8166 cells	
Primary					
2005	SI	NT ^a	4.6 × 10 ⁴	1.3 × 10 ⁵	0.4
2006	SI	1.3 × 10 ³	4.6 × 10 ³	1.3 × 10 ²	35
2028	SI	6.3 × 10 ⁴	4.4 × 10 ³	2.9 × 10 ³	1.5
2029	SI	6.3 × 10 ³	5.3 × 10 ³	2.9 × 10 ⁴	0.2
2036	SI	6.3 × 10 ³	6.4 × 10 ²	2.9 × 10 ⁴	0.02
2044	SI	3.0 × 10 ⁴	6.9 × 10 ³	6.3 × 10 ⁴	0.1
2053	SI	6.3 × 10 ³	3.0 × 10 ¹	2.9 × 10 ²	0.1
2075	SI	1.3 × 10 ³	1.5 × 10 ³	1.3 × 10 ³	1.2
2076	SI	3.0 × 10 ⁵	7.4 × 10 ⁴	1.3 × 10 ⁵	0.6
3028	SI	1.3 × 10 ³	2.1 × 10 ³	2.9 × 10 ²	7.2
M13	SI	2.0 × 10 ³	8.5 × 10 ²	1.3 × 10 ²	6.5
89.6	SI	5.0 × 10 ³	6.3 × 10 ³	5.0 × 10 ⁴	0.1
E80	NSI	2.0 × 10 ⁴	2.5 × 10 ⁴	<1.3 × 10 ¹	>1.9 × 10 ³
M23	NSI	1.3 × 10 ¹	1.4 × 10 ⁴	<1.3 × 10 ¹	>1.1 × 10 ³
SL-2	NSI	3.0 × 10 ²	1.0 × 10 ³	<1.3 × 10 ¹	>77
SL-3	NSI	2.9 × 10 ¹	5.0 × 10 ²	<1.3 × 10 ¹	>38
SL-4	NSI	1.3 × 10 ¹	5.0 × 10 ²	<1.3 × 10 ¹	>38
SF162	NSI	2.0 × 10 ³	1.8 × 10 ⁵	<1.3 × 10 ¹	>1.3 × 10 ⁴
T-cell line adapted					
GUN-1	SI	NT	6.0 × 10 ⁴	4.6 × 10 ⁷	0.001
RF	SI	6.8 × 10 ⁵	2.0 × 10 ²	6.4 × 10 ⁸	3.3 × 10 ⁻⁷
LAI	SI	2.0 × 10 ⁵	5.0 × 10 ¹	6.4 × 10 ⁷	7.8 × 10 ⁻⁷

^a NT, not tested.

than SF-162 (23). Primary strains M13, M23, E80, SL-2, SL-3, and SL-4 were isolated at St. Mary's Hospital, London, England. SL-2, SL-3, and SL-4 were from asymptomatic patients from Thailand. M13, M23, and E80 were from British patients attending the St. Mary's Hospital STD clinic. Isolates 2005, 2006, 2028, 2029, 2036, 2044, 2053, 2075, 2076, and 3028 were made at Addenbrooke's Hospital, Cambridge, England, from patients whose CD4⁺ blood cell counts were less than 190 cells mm⁻³ except for the patients from whom isolates 2076 and 3028 were derived; these individuals had CD4 counts of 400 and 328, respectively. Isolates 2005, 2029, and 2036 were from patients with AIDS. Isolates made in Cambridge were from patients registered in Birmingham (2006), Hull (2005 and 2075), London (2036, 2044, 2053, and 2076), and Portsmouth (2028 and 2029), England, and Uganda (3028). All isolates were of subtype B except for 3028, M13, M23, and E80, which were unclassified. Isolates were cultured from phytohemagglutinin-interleukin-2-stimulated PBMCs derived from the peripheral blood of infected individuals. Freshly cultured PBMCs from HIV-negative donors were added every 4 to 8 days. Supernatants were harvested twice weekly and screened for p24 antigen. Positive supernatants were aliquoted and stored in liquid nitrogen. All primary strains were minimally passaged in PBMCs to prepare virus stocks.

Biological clones of viruses were made by limiting dilution. Fivefold dilutions of virus supernatant were added to PBMC cultures on 96-well trays. Virus was rescued and propagated from p24⁺ wells, of which less than 10% of wells per tray were positive. Plasmid DNA containing molecularly cloned virus was transfected directly into either PBMCs or C8166 cells by using Lipofectamine (Gibco BRL) as indicated by the manufacturer before preparation of virus stocks.

Infectivity assays. Macrophages and PBMCs were infected with appropriate HIV-1 strains 5 to 6 days after establishment as cultures. Macrophages were exposed to half-log dilutions of virus for 2 h, washed once, and cultured in RPMI 1640 containing 10% human serum and 5% fetal calf serum for 21 days. Each virus strain was tested for infection of three batches of macrophages prepared from separate donors. PBMCs and C8166 cells were adhered to plastic with poly-L-lysine for 1 h, infected for 2 h, washed, and overlaid with growth medium containing low-viscosity (3 mg/ml) and high-viscosity (3 mg/ml) carboxymethyl-cellulose. C8166 cells were cultured for 7 days and PBMCs were cultured for 9 days before immunostaining for p24 antigen as previously described (8).

Transient expression of Lestr and CCR5 on CCC/CD4 cells. Three and a half micrograms of plasmid pcDNA3.1 plasmid (Invitrogen) containing cDNA clones of either Lestr and CCR5 was transfected into CCC/CD4 cat cells, using Lipofectamine as described above. A total of 7.5×10^4 transfected cells were challenged for virus infection 48 h later.

RESULTS

Infectivity of primary SI, NSI, and T-cell line-adapted HIV-1 strains for CD4⁺ T-cell lines and for PBMC and macrophage cultures. Eleven primary SI and five NSI HIV-1 isolates were

assayed for infection of the CD4⁺ T-cell line C8166 as well as primary PBMC and macrophage cell cultures. T-cell line-adapted strains RF and LAI were tested for comparison, as was the T-cell line-passaged dual-tropic strain GUN-1. SF-162 and 89.6 were also included as well-characterized, molecularly cloned NSI/macrophage-tropic and SI/dual-tropic strains, respectively (5, 12). All primary SI and T-cell line-adapted SI strains replicated and induced syncytia in MT-2 cells, whereas none of the NSI strains did (data not shown). Table 1 shows virus infectivity titers (50% tissue culture infective doses [TCID₅₀]) for each cell type. Infectivity titers on primary macrophages were compared with those on C8166 cells and expressed as a ratio for each virus strain tested. T-cell line-adapted strains RF and LAI infected PBMCs and C8166 cells to high titers but did not to infect macrophages efficiently. Macrophage/C8166 infectivity ratios for these viruses are therefore very low (3.3×10^{-7} to 7.8×10^{-7}). All primary SI isolates infected PBMCs and C8166 cells and showed variation in the capacity to infect macrophages, as illustrated by their macrophage/C8166 infectivity ratios, which varied from 0.02 to 35. However, all ratios for primary SI viruses were several orders of magnitude higher than ratios for T-cell line-adapted viruses. Thus, primary SI strains are consistently more efficient for macrophage infection than T-cell line-adapted viruses. Some isolates, e.g., 2005 and 2076, infect both C8166 cells and macrophages efficiently and are thus dual-tropic, whereas others, e.g., 2053, infect macrophages only to levels similar to those of LAI (Table 1). Such strains, however, are more macrophage-tropic than LAI since they yield lower titers on PBMCs and C8166 cells. Each of the six NSI strains, including SF-162, replicated more efficiently in macrophages than in PBMCs. The differences between primary SI and T-cell line-adapted SI strains was also apparent when we plotted macrophage/PBMC infectivity ratios (Fig. 1). Such ratios for four of six primary NSI strains were highest, suggesting that these

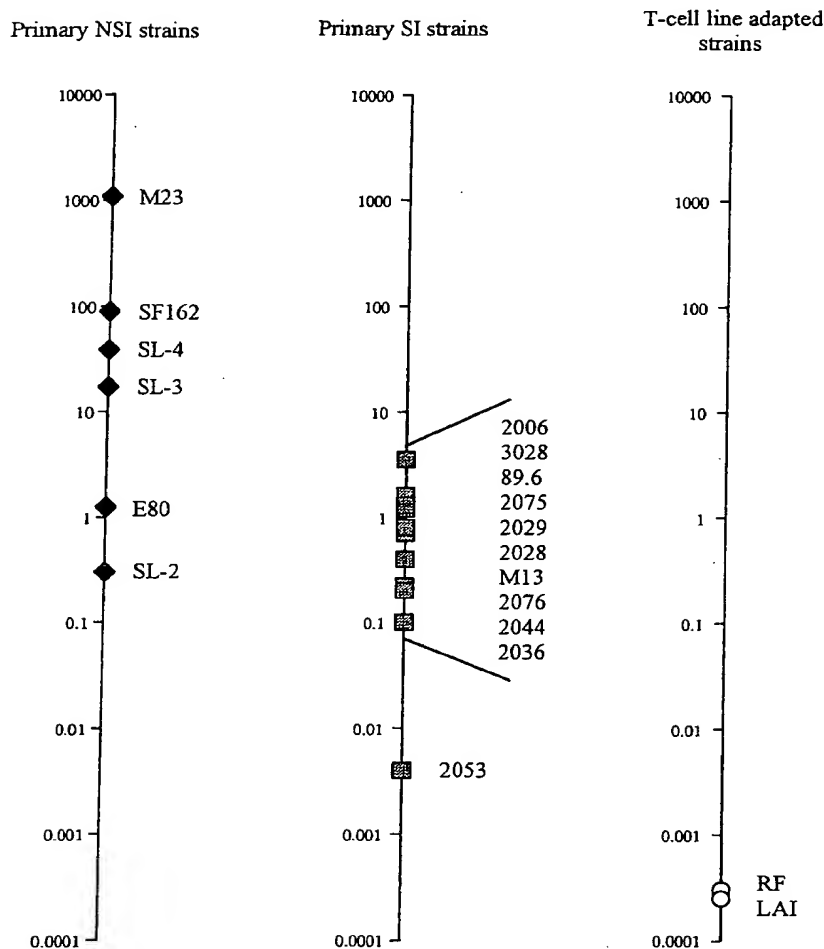


FIG. 1. Macrophage/PBMC infectivity ratios for primary and T-cell line-adapted HIV-1 strains. HIV-1 infectivity titers for primary macrophage and PBMC cultures (taken from Table 1) were used to calculate macrophage PBMC infectivity ratios, which are plotted for each HIV-1 isolate used.

strains were the most tropic for macrophages (however, see Discussion).

Biological and molecular clones of primary SI strains are dual-tropic. As the dual-tropism of some of the primary SI isolates could be due to a mixture of viruses with different phenotypes within a single isolate, two of the dual-tropic isolates were biologically cloned by limiting dilution (see Materials and Methods). Table 2 shows the titers for five clones of 2076 and four clones of 2005. In all cases, the cloned viruses retained the parental dual-tropic phenotype, strongly suggesting that their dual-tropic phenotype does not result from a mixture of macrophage-tropic NSI and T-cell tropic SI viruses. It therefore seems likely that the dual-tropic/SI phenotypes of 89.6 and GUN-1 viruses, which are derived from molecular DNA clones, are more representative of primary SI isolates than are T-cell line-adapted LAI and RF.

Coreceptors used by primary SI viruses. Recently Lestr (19) and the chemokine receptor CCR5 (1, 15, 17), members of the extensive family of seven-transmembrane, G-protein-coupled receptors, have been identified as coreceptors for SI, T-cell line-adapted HIV-1 strains and NSI, macrophage-tropic viruses, respectively. We tested whether either of these corecep-

TABLE 2. Infectivity of biological clones made from HIV-1 primary SI isolates

Virus isolate	Infectivity titer (TCID ₅₀ /ml)		Ratio, macrophage infectivity/C8166 cell infectivity
	Macrophages	C8166 cells	
2076			
Parental	7.4×10^4	1.3×10^5	0.6
Clones			
1	8.0×10^4	3.6×10^5	0.2
2	8.0×10^3	2.6×10^3	3.0
3	5.0×10^3	8.0×10^3	0.6
4	1.1×10^3	3.6×10^3	0.3
5	3.6×10^4	1.6×10^5	0.2
2005			
Parental	4.6×10^4	1.3×10^5	0.4
Clones			
1	1.6×10^3	5.0×10^4	0.03
2	1.1×10^3	3.6×10^3	0.3
3	2.6×10^2	1.6×10^3	0.16
4	1.1×10^3	8.0×10^3	0.14

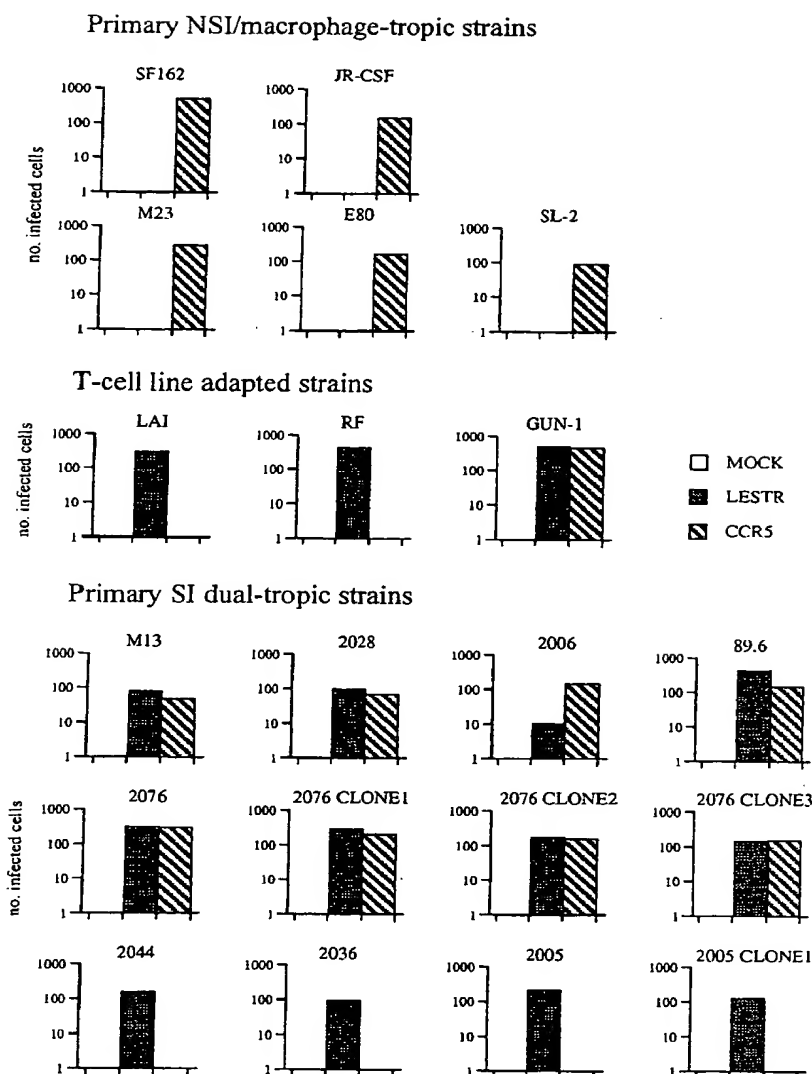


FIG. 2. Infection of CCC/CD4 cat cells expressing either Lestr or CCR5 by primary NSI and SI HIV-1 strains. Two hundred to 1,000 TCID₅₀ for PBMC or macrophages (whatever was highest) was added to 7.5×10^4 CCC/CD4 cells expressing either Lestr or CCR5. The number of infected cells, estimated following 4 days culture and immunostaining for p24, is shown for each transfectant. Untransfected CCC/CD4 cells were resistant to infection by all strains tested.

tors functioned for primary dual-tropic SI viruses. Lestr or CCR5, cloned into the expression plasmid pcDNA3.1, was transiently expressed in CCC/CD4 cells following transfection of plasmid DNA by Lipofectamine. CCC/CD4 cells are resistant to HIV-1 but permissive to entry and productive replication by several T-cell line-adapted HIV-2 strains (7, 25). Forty-eight hours after transfection, the CCC/CD4 cells were challenged with seven primary SI viruses and three primary NSI viruses as well as by standard T-cell line-adapted viruses and viruses derived from molecular clones (NSI strain JR-CSF [23], NSI/macrophage-tropic strain SF-162 [5], and the dual-tropic SI strains 89.6 [12] and GUN-1 [38]). After 4 days of incubation, cells were fixed and stained for p24 antigen as described in Materials and Methods. Figure 2 shows that T-cell line-adapted viruses LAI and RF infected only Lestr⁺ CCC/CD4 cells, whereas primary NSI viruses M23, E80, SL-2,

and SF-162 infected only CCR5⁺ CCC/CD4 cells. In contrast, four of the primary dual-tropic SI viruses, 2006, M13, 2028, and 2076 (as well as three biological clones of 2076), infected both Lestr⁺ and CCR5⁺ CCC/CD4 cells. Furthermore, the two dual-tropic viruses derived from molecular DNA clones (GUN-1 and 89.6) also infected both Lestr⁺ and CCR5⁺ CCC/CD4 cells. Thus, these primary SI strains of HIV-1 are dual-tropic and can use either Lestr or CCR5 as coreceptors. Interestingly, three other primary SI strains (2044, 2036, and 2005), each of which infected macrophage cultures (Table 1), infected only Lestr⁺ CCC/CD4 cells and not CCR5⁺ CCC/CD4 cells.

DISCUSSION

HIV-1 strains that have been passaged extensively in CD4⁺ T-cell lines, e.g., LAI and RF, usually infect primary macro-

phage cultures inefficiently (11, 37). Here, we tested whether macrophage infection by primary SI isolates was comparable to that of T-cell line-adapted strains, i.e., whether the SI phenotype correlated with inefficient infection of macrophages. Our results indicate that macrophage infection by primary SI strains is highly variable but that all strains tested infected macrophages more efficiently than the T-cell line-adapted strains LAI and RF. Macrophage infection by the six NSI strains tested was also highly variable (Table 1), yet in contrast to the SI strains, these viruses consistently infected macrophages more efficiently than PBMC cultures (Fig. 1). Our study therefore suggests that efficiency of macrophage replication follows the order primary NSI > primary SI >> T-cell line-adapted SI strains. Yet, it is likely that the titers for NSI/macrophage-tropic viruses recorded on PBMC cultures are artificially low as a result of inhibitory chemokines, e.g., RANTES, produced by lymphocytes (10, 17, 32), thus overestimating the macrophage-tropism of such strains.

The strain differences reported above are attributable at least in part to differences in coreceptor usage. We transiently expressed either Lestr or CCR5 on CCC/CD4 cat cells and tested for infection by representative NSI and SI primary strains. CCC/CD4 cells resist HIV-1 infection but are fully permissive to productive replication by several HIV-2 strains (25). Our results show clearly that dual-tropic viruses derived from DNA molecular clones as well as several primary SI viruses (M13, 2028, 2006, and 2076) use either Lestr or CCR5. In contrast, T-cell line-adapted HIV-1 strains infected Lestr⁺ cells only and NSI strains infected CCR5⁺ cells only, as previously reported (1, 15, 17, 19). Some primary SI strains (2005, 2036, and 2044) that infected macrophages (Table 1) infected Lestr⁺ CCC/CD4 cells but not CCR5⁺ CCC/CD4 cells. Presumably these strains use a coreceptor different than CCR5 for infection of macrophages. However, at this stage we cannot exclude the possibility that CCR5 is differently presented or processed on CCC/CD4 cells so that entry of some strains but not others is affected.

We were concerned that the dual-tropic phenotype of primary SI strains could be caused by a mixture of NSI/macrophage-tropic and SI/T-cell tropic strains in the original isolate. However, biological clones prepared from two primary SI strains each maintained a dual-tropic phenotype for macrophages and T-cell lines (Table 1). Clones derived from isolate 2076 infected both Lestr⁺ and CCR5⁺ CCC/CD4 cells, whereas clones derived from 2005 infected only Lestr⁺ CCC/CD4 cells. Thus, each biological clone retained the phenotype of the parental virus. These observations, including infection of either Lestr⁺ or CCR5⁺ CCC/CD4 cells by the 2076 subclones and by the GUN-1 and 89.6 molecular clones, show that at least some HIV-1 strains can use more than one coreceptor and can choose between compatible coreceptors for entry. Indeed, recently we demonstrated that the HIV-2_{ROD} envelope glycoproteins could use a number of CC and CX_C chemokine receptors to induce cell-cell fusion of CD4⁺ cells, although only some supported efficient cell-free virus entry (3).

The difference between T-cell line-adapted strains and primary SI strains described above is likely to be caused by selection against CCR5 use by extensive in vitro passage in Lestr⁺ CCR5⁻ T-cell lines. Most CD4⁺ T-cell lines used for in vitro passage of HIV resist infection by primary NSI strains and are thus likely to express lower levels of CCR5 or no CCR5 (35). Extensive passage of SI strains in such lines is therefore likely to select for more efficient use of Lestr against CCR5 for virus entry.

Our results show conclusively that Lestr can also act as a coreceptor for primary SI viruses. Every primary SI virus tested

infected Lestr⁺ CCC/CD4 cells. Lestr is thus likely to play an important role in HIV-1 pathogenesis and in the rapid decline of CD4⁺ T-cells that occurs after emergence of SI strains in vivo. SI strains can be isolated from only about 50% of patients with AIDS, and it will therefore be interesting to investigate whether a change in coreceptor use might also occur for rapid/high NSI viruses prevalent in these patients (14).

The discovery of the nature of coreceptors for HIV-1 entry opens up the possibility of designing inhibitors to block their interaction with HIV envelope glycoproteins. RANTES, MIP-1 α , and MIP-1 β all bind to CCR5 and block entry of NSI/macrophage-tropic HIV-1 strains (10). Furthermore, lymphocytes from multiply exposed yet HIV-seronegative individuals produce high levels of these chemokines (32). RANTES, MIP-1 α , and MIP-1 β , however, are not specific for CCR5 and bind other chemokine receptors too (4, 13, 30, 34, 36). Recently, we reported that a monoclonal antibody (12G5) that recognizes Lestr but not other chemokine receptors blocked fusion of T-cell line-adapted HIV-1 strains with some Lestr⁺ cells but not others (27). Either alternative unidentified coreceptors allowed escape from 12G5 or Lestr might be processed or presented differently to enable escape from 12G5 blocking. Whatever the reason, these observations indicate that design of inhibitors directed to specific chemokine or coreceptor molecules for HIV inhibition is a new challenge.

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Dual Tropism for Macrophages and Lymphocytes Is a Common Feature of Primary Human Immunodeficiency Virus Type 1 and 2 Isolates

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We have investigated the ability of human immunodeficiency virus type 1 (HIV-1) and HIV-2 isolates to infect and replicate in primary human macrophages. Monocytes from blood donors were allowed to differentiate into macrophages by culture in the presence of autologous lymphocytes and human serum for 5 days before infection. A panel of 70 HIV-1 and 12 HIV-2 isolates were recovered from seropositive individuals with different severities of HIV infection. A majority of isolates (55 HIV-1 and all HIV-2) were obtained from peripheral blood mononuclear cells, but isolates from cerebrospinal fluid, monocytes, brain tissue, plasma, and purified CD4⁺ lymphocytes were also included. All isolates were able to infect monocyte-derived macrophages, even though the replicative capacity of the isolates varied. Interestingly, isolates with a rapid/high, syncytium-inducing phenotype did not differ from slow/low, non-syncytium-inducing isolates in their ability to replicate in monocyte-derived macrophages. Others have reported that rapid/high, syncytium-inducing isolates have a reduced ability to infect and replicate in monocytes. However, different methods to isolate and culture the monocytes/macrophages were used in these studies and our study. Thus, the ability of HIV isolates to replicate in monocytes/macrophages appears to be strongly influenced by the isolation and culture procedures. It remains to be determined which culture procedure is more relevant for the *in vivo* situation.

Human immunodeficiency virus (HIV) isolates vary in biological properties such as host range, replicative rate, and capacity to induce cytopathic changes. These biological properties correlate with the severity of HIV infection (3, 6, 9, 30). HIV isolates from individuals with mild or no symptoms usually lack the capacity to grow in established cell lines and induce only small or no syncytia; such isolates have been called slow/low (3, 9) or non-syncytium inducing (30). In contrast, many HIV isolates from patients with severe immunodeficiency can replicate in CD4⁺ cell lines and induce large syncytia in culture; these isolates are referred to as rapid/high (3, 9) or syncytium inducing (30). Furthermore, it has been proposed that HIV variants can be distinguished according to their capacity to infect primary mononuclear phagocytes or T-helper lymphocytes (5, 14, 28, 34). Some HIV variants were shown to lack the capacity to infect monocyte-derived macrophages (MDM), while others were found to be dually tropic for both T lymphocytes and mononuclear phagocytes (14, 34). Passage of HIV isolates through phytohemagglutinin (PHA)-P-stimulated peripheral blood lymphocytes (PBL) yielded progeny virus unable to infect MDM (14). Monocytotropic HIV variants have been reported to have a non-syncytium-inducing phenotype and thus lack the capacity both to replicate in T-cell lines and to induce syncytia in primary lymphocyte cultures (28). In contrast, results obtained by other groups, including ours, suggest that tropism for mononuclear phagocytes is a general property of all primary HIV type 1 (HIV-1)

isolates (7, 13, 31, 32) and that repeated passage of an HIV-1 isolate through PBL does not alter its capacity to infect MDM (13).

Differences in replicative capacity in MDM have been suggested to be an important selective factor during transmission of HIV-1 (36). Thus, it is of great importance to clarify if certain HIV-1 variants lack the ability to infect mononuclear phagocytes. In this study, we have tested the ability of a panel of well-characterized HIV-1 and HIV-2 isolates to replicate in MDM that were obtained from peripheral blood mononuclear cells (PBMC) by plastic adherence for 5 days in the presence of autologous lymphocytes and human serum. In addition, we investigated if the tropism of HIV for MDM is related to other biological properties, such as cytopathicity or replicative capacity in established cell lines. Our results show that all HIV isolates can infect and replicate in primary mononuclear phagocytes, regardless of differences in biological phenotype.

MATERIALS AND METHODS

Cells. MDM were prepared from heparinized venous blood from healthy HIV-negative donors by plastic adherence for 5 days according to the method of Gartner et al. (12). Briefly, PBMC obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) separation were washed five times with phosphate-buffered saline (PBS) (the last wash was made at 800 rpm to remove platelets). PBMC were seeded in 25-cm² tissue culture flasks (25 × 10⁶ PBMC) or in 9-cm² tissue culture slide flasks (9 × 10⁶ PBMC) in the presence of RPMI 1640 culture medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with L-glutamine, penicillin (50 U/ml), streptomycin (50 µg/ml), 20% heat-inactivated fetal calf serum, and 10% heat-inactivated pooled human HIV-1-negative serum; PBMC were cultured at 37°C in 5% CO₂. Five days after initiation of the cultures, nonadherent cells were removed by rinsing the

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cultures three times with PBS. Adherent cells were grown in the same medium without HIV-1-negative serum. The cells obtained by this method are esterase positive and trypsin resistant and show a typical monocyte/macrophage phenotype, with expression of receptors for the Fc fragment of immunoglobulin G, complement receptors, and CD14, a marker for monocytes among the mononuclear cell population (12). No cells positive for CD2 or CD3 were present in the cultures.

PBL were obtained from PBMC from healthy blood donors by stimulation for 3 days with 2.5 μ g of PHA per ml. PBL were cultured in RPMI medium containing 10% fetal calf serum, interleukin-2, and antibiotics as previously described (1). Purified CD4⁺ lymphocytes were obtained from PBMC by the use of magnetic beads (Dynal, Oslo, Norway) coated with a monoclonal antibody against CD4 (4). The cells were kindly provided by J. Brinchmann at the Institute of Transplantation Immunology, National Hospital, Oslo, Norway. The human monocytoid cell line U937 and the human T-cell line CEM were cultured in RPMI medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics.

Viruses. A panel of 70 HIV-1 and 12 HIV-2 primary isolates was used. All HIV-2 and 55 HIV-1 isolates were obtained by cocultivation of patients' PBMC with PHA-stimulated PBL from uninfected donors as described previously (1, 2). Remaining HIV-1 isolates were obtained from the following sources: two from cerebrospinal fluid (recovered in PBL), three from patients' monocytes, two from brain tissue (recovered in PBL), four from plasma (two recovered in PBL and two recovered in MDM), and four directly isolated from purified CD4⁺ T lymphocytes (two in the absence and two in the presence of the patients' own CD8⁺ cells) (4). The latter four isolates were kindly provided by J. Brinchmann. Sixteen HIV-1 isolates were obtained from asymptomatic individuals, 11 were obtained from patients with persistent generalized lymphadenopathy, 17 were obtained from patients with AIDS-related complex or constitutional disease, and 10 were obtained from patients with AIDS. The clinical stage of the remaining 16 patients (some of them children from the former USSR accidentally infected with HIV-1-contaminated needles) was unknown. In the case of HIV-2 isolates, three isolates were from asymptomatic individuals, two were from patients suffering from severe asthenia, two were from patients with tuberculosis or pulmonary disease, and five were from patients with AIDS according to the Bangui criteria. A majority of the isolates used in the study have been biologically characterized (2, 9): 22 HIV-1 and 4 HIV-2 isolates grow well in human cell lines and induce syncytia (rapid/high phenotype), whereas 39 HIV-1 and 8 HIV-2 isolates lack the capacity to continuously replicate in established cell lines and induce no syncytia (slow/low phenotype).

HIV infection. The MDM cultures were infected 7 days after initiation. Prior to infection, the MDM were washed once with PBS. In addition, in the experiments with the laboratory strains IIIB and BaL, the cells had been vigorously trypsinized 5 days after initiation of the cultures (2 days before infection). Virus inocula for all infections ranged between 3×10^4 and 5×10^4 cpm of reverse transcriptase (RT) activity. After addition of the virus, the cells were incubated overnight at 37°C in 5% CO₂. The next day, the cells were washed two or three times with PBS and once with RPMI 1640 to remove unadsorbed virus, and fresh medium was added. The culture medium was changed once weekly in the MDM cultures and twice weekly in the PBL and cell line cultures; the latter were also split when required. Supernatants were stored for determinations of RT activity and levels of p24 antigen. If the cultures were negative by RT assay and p24 antigen assay after 4 weeks, attempts were

made to rescue virus production by adding 10×10^6 PHA-stimulated blood donor PBL to the cultures.

RT assay. For the RT assay, 1.0 ml of cell-free culture supernatant was collected and centrifuged ($15,000 \times g$, 1 h) (29). The pellet was resuspended in 100 μ l of lysis buffer containing 0.3% Triton X-100, 75 μ M Tris-HCl (pH 8.0), 50 mM KCl, and 6 mM dithiothreitol. Fifty microliters of this mixture was analyzed. The RT reaction mixture (100 μ l) contained 75 μ M Tris-HCl (pH 8.0), 50 mM KCl, 6 mM dithiothreitol, 6 mM MgCl₂, 100 μ g of bovine serum albumin per ml, 2.5 μ g of oligo(dT)₁₂₋₁₄ (Pharmacia) per ml, 2.0 μ g of poly(A) (Pharmacia) per ml, and 25 μ Ci of [³H]dTTP per ml (50 Ci/mmol; DuPont). Cultures were considered RT positive if two consecutive samples gave RT assay values above the background (>3,000 cpm).

HIV antigen assay. HIV-1 p24 antigen levels were determined by an in-house enzyme-linked immunosorbent assay (ELISA) (29) as previously described. Microtiter plates (Nunc I, Roskilde, Denmark) were coated with immunoglobulins (20 μ g/ml) prepared from the serum of an asymptomatic HIV-1-infected person with high levels of anti-HIV-1 antibodies. Two mouse monoclonal antibodies reacting with different epitopes of the HIV-1 p24 antigen were used as tracing antibodies. The tracing antibodies had been directly conjugated to horseradish peroxidase. The detection limit of the p24 antigen assay is 50 to 100 pg of p24 antigen per ml (29). The sensitivity of the RT assay has been shown to be 5 to 10 times lower than that of the p24 antigen assay (29). Cultures were considered HIV antigen positive if two consecutive samples gave increasing absorbance values above the background ($A_{490} > 0.3$). HIV-2 antigen samples were assayed by a commercial HIV-1 antigen ELISA (Abbott) which cross-reacts with HIV-2.

RESULTS

Infection of MDM with HIV isolates. MDM were infected with a panel of well-characterized HIV isolates obtained from patients with different severities of HIV infection and from different tissue and cell origins. Sixty HIV-1 and 9 HIV-2 isolates were primary isolates, whereas 10 HIV-1 and 3 HIV-2 isolates had been passaged in PHA-stimulated PBL. Virus replication was monitored by RT activity and the presence of p24 antigen in culture supernatants. In two cultures, which did not show any detectable virus production, PHA-stimulated PBL were added to the MDM cultures after 4 weeks. As shown in Table 1, all HIV isolates could infect the MDM cultures, even if they had been previously passaged in PBL. However, the rates of replication varied among the isolates. Virus production could be demonstrated by RT activity in supernatants of 52 cultures and by the more sensitive p24 antigen assay in an additional 28 cultures. Only two MDM cultures were negative by both assays, but virus infection could also be demonstrated in these two cultures by cocultivation with PBL.

The ability of the isolates to replicate in MDM was analyzed semiquantitatively by dividing them into two groups: isolates that were positive by RT assay and isolates that were positive only by the more sensitive p24 antigen assay and coculture techniques. Interestingly, there were no significant differences in the replicative capacities in MDM between HIV isolates with a slow/low phenotype (31 of 47 RT-positive cultures) and isolates with a rapid/high phenotype (15 of 26 RT-positive cultures) ($\chi^2 = 0.2$, not significant) (Table 2). Similarly, replicative capacities of isolates obtained from patients with mild or no symptoms did not differ significantly (23 of 32 RT-positive cultures) from those of isolates derived from patients with more severe disease (18 of 34 RT-positive

TABLE 1. Infection of MDM by primary HIV isolates and PBL-passaged viruses

Virus	No. of positive cultures detected by:			Total no. positive
	RT assay	p24 assay ^a	Cocultivation ^b	
HIV-1				
Primary isolates	35	23	2	60
PBL passaged	9	1		10
HIV-2				
Primary isolates	5	4		9
PBL passaged	3			3
Total	52	28	2	82

^a Number of HIV isolates in which virus replication was detected by the p24 antigen ELISA but not by the less sensitive RT assay.

^b Number of HIV isolates in which virus replication was detected by cocultivation with PHA-stimulated PBL but not by the less sensitive RT assay or by the p24 antigen ELISA.

cultures) ($\chi^2 = 1.77$, not significant) (data not shown). It is noteworthy that infection with most of the PBL-passaged isolates (12 of 13) could be detected by RT activity in culture supernatant, while this was the case for only 40 of 69 primary isolates.

MDM infection with HIV isolates from purified CD4⁺ lymphocytes. Next we investigated if HIV isolates obtained from purified T-helper lymphocytes and selectively passaged in this cell type retained the capacity to infect and replicate in human mononuclear phagocytes. These isolates were passaged four times in monocyte-free CD4 lymphocytes purified from blood donors. Biological characterization of these viruses demonstrated that isolates 1 and 2 have a slow/low phenotype and isolates 3 and 4 have rapid/high properties (data not shown). All isolates could infect and replicate in MDM (Table 3). There were no significant differences in replication kinetics among the isolates, except that isolate 4 was positive only by p24 antigen assay 3 weeks after infection.

MDM infection with HIV-1_{IIIB} and HIV-1_{BaL}. In the literature, there are conflicting data about the capacity of HIV-1_{IIIB} to infect human mononuclear phagocytes (15, 19, 28). Therefore, we compared the capacities of HIV-1_{IIIB} and HIV-1_{BaL}.

TABLE 2. Infection of MDM by HIV isolates with different biological properties

Virus	No. of positive cultures detected by:			Total no. positive
	RT assay ^a	p24 assay ^b	Cocultivation ^c	
Slow/low				
HIV-1	26	11	2	39
HIV-2	5	3		8
Rapid/high				
HIV-1	12	10		22
HIV-2	3	1		4
Total	46	25	2	73

^a Number of HIV isolates that replicated to high levels in MDM as detected by RT activity in culture supernatant. The replicative capacities of the different isolates varied but with no significant differences between HIV-1 and HIV-2 isolates. The mean value for maximal RT activity for slow/low isolates was 73×10^3 cpm/ml, with a range from 520×10^3 to 8×10^3 cpm/ml. In the case of rapid/high isolates, the mean was 67×10^3 cpm/ml, and the range was from 206×10^3 to 8×10^3 cpm/ml.

^b See footnote a in Table 1.

^c See footnote b in Table 1.

TABLE 3. Replication of CD4⁺ lymphocyte-derived HIV-1 isolates in MDM

Virus	RT activity (10^3 cpm/ml)		
	Day 7	Day 14	Day 21
1	96	135	85
2	78	87	94
3	43	67	38
4	32	17	— ^a

^a —, a value lower than five times the background level (0.5×10^3 cpm/ml). This culture was positive for p24 antigen (10 ng/ml).

to infect and replicate in MDM and PHA-stimulated PBMC. Stocks of both viruses were serially diluted (10-fold) in RPMI 1640 culture medium. Table 4 shows that HIV-1_{BaL} replicated to higher levels than HIV-1_{IIIB} in MDM but the end-point dilution for a productive infection of MDM was the same for both viruses in two independent experiments and one step lower for HIV-1_{BaL} in a third experiment (data not shown). We also tested if HIV-1_{IIIB} passaged in PBL or cell lines (H9 and Jurkat) differed in the ability to infect human macrophages. MDM were infected with 4×10^4 cpm of RT activity of HIV-1_{IIIB} obtained from PBL and H9 and Jurkat cells. In our hands, all three HIV-1_{IIIB} stocks could infect and replicate in MDM irrespective of the cellular origin (data not shown). In order to exclude cross-contamination of the HIV-1_{IIIB}-infected MDM cultures, we determined the RNA sequence of virus particles produced by a MDM culture infected by HIV-1_{IIIB} derived from PBL. Thus, virion-associated RNA was extracted, reverse transcribed into cDNA, PCR amplified, and sequenced by previously described methods (25). As expected, the V3 loop sequence of the virus produced by the HIV-1_{IIIB}-infected MDM culture was identical to the published sequence of this virus isolate (data not shown).

Biological characterization of HIV strains after passage in PBL and MDM cultures. HIV-1 has been reported to consist

TABLE 4. Infection of MDM and PBL with serial dilutions of HIV-1_{IIIB} and HIV-1_{BaL}^a

Strain and dilution	PBL		MDM	
	Max RT activity (10^3 cpm/ml)	p24 ^b	Max RT activity (10^3 cpm/ml)	p24
IIIB				
None	560		43	
10^{-1}	222		34	
10^{-2}	148		14	
10^{-3}	112		7	
10^{-4}	66		— ^c	+
10^{-5}	54		—	+
10^{-6}	—	—	—	—
BaL				
None	119		201	
10^{-1}	103		179	
10^{-2}	106		66	
10^{-3}	37		19	
10^{-4}	26		12	
10^{-5}	—	—	—	+
10^{-6}	—	—	—	—

^a MDM and PBL were infected with serial 10-fold dilutions of supernatants containing 100×10^3 cpm of RT activity of HIV-1_{IIIB} and HIV-1_{BaL} per ml.

^b Only cultures considered negative for RT activity were tested for the presence of the p24 antigen by ELISA.

^c —, a value considered negative for RT activity.

TABLE 5. Biological characterization of HIV strains after passage in PBL and MDM

Passage cell type and virus ^a	Max RT activity (10 ³ cpm/ml) in:		
	PBL	U937	CEM
PBL			
IIIB	608	413	167
A692	1,018	238	4
6669	417	29	— ^b
MDM			
IIIB	650	460	83
A692	397	93	7
6669	387	376	—

^a The infectious supernatants used in these experiments were obtained after in vitro propagation of the different HIV strains in PBL and MDM. Syncytium formation, which was observed in all strains regardless of passage cell type, was evaluated in PBL cultures.

^b —, a value considered negative for RT activity.

of a mixture of variants with different biological properties (27); therefore, it is conceivable that passage of an HIV isolate in MDM may select for a minor virus population with properties distinct from those of the original isolate. To test this possibility, PHA-stimulated PBL and MDM were infected in parallel with three HIV strains (HIV-1_{IIIB}, HIV-1_{A692}, and HIV-2_{SBL6669}) which all replicate in CD4⁺ cell lines and induce syncytium formation. Progeny virus from these cultures were used as cell-free supernatant to infect PBL, the monocytoïd cell line U937, and the T-cell line CEM. Results from these experiments are shown in Table 5. Phenotypic changes were not observed for any of the HIV strains after in vitro passage in MDM or PBL. All three strains were also still able to replicate in cell lines and induce syncytium formation after passage in MDM.

DISCUSSION

The major targets for HIV infection are bone marrow-derived cells expressing CD4 on their surfaces. HIV infection in vivo has been demonstrated in T-helper lymphocytes (17) and mononuclear phagocytes (8, 18, 22, 23, 35). However, there is considerable controversy about whether all or only certain HIV isolates have the ability to replicate in monocytes/macrophages (5, 7, 13, 14, 28, 31, 32, 34). For this reason and since monocytes/macrophages are the main target cells for other lentiviruses (20), we systematically investigated the capacity of well-characterized HIV-1 and HIV-2 isolates to infect and replicate in primary human MDM. The MDM were derived from blood donor PBMC by plastic adherence for 5 days in the presence of autologous lymphocytes and human serum. Our results show that all HIV isolates could infect these cells, although the levels of replication varied.

As indicated above, several authors have previously reported that only certain HIV-1 isolates can productively infect monocytes/macrophages (5, 14, 28, 34). Thus, Schuitemaker et al. (28) reported that most syncytium-inducing HIV isolates do not replicate in macrophages. Furthermore, Gendelman et al. (14) reported that serial passage of an HIV isolate in PBL selects for virus variants lacking the capacity to infect mononuclear phagocytes. In contrast, other groups, including ours, have found that all HIV-1 isolates can productively infect MDM (7, 13, 31, 32). In this study, we have directly tested if the biological phenotype and passage history of HIV isolates influence the ability to replicate in MDM. In contrast to

Schuitemaker et al. (28) and Gendelman et al. (14), we found that isolates with a rapid/high, syncytium-inducing phenotype can infect and replicate in MDM and that the biological phenotype of HIV isolates remains unchanged after passage in MDM and purified CD4⁺ lymphocytes. In support of our findings, Connor et al. (7) recently reported that all 28 HIV-1 isolates sequentially obtained from four individuals replicated in macrophages, despite differences in their ability to replicate in tumor cell lines. Similarly, Gartner and Popovic (13) reported that repeated passage of an HIV-1 isolate through MDM does not generally result in the loss of the ability to infect normal T cells. In agreement with results from earlier reports, our results were that the BaL isolate of HIV-1 differed from the IIIB isolate in that it replicated to higher titers on MDM than on PBL (12, 28).

The seemingly conflicting data on the ability of HIV-1 isolates to replicate in monocytes/macrophages are probably due to technical differences in the methods used to isolate and cultivate the macrophages and monocytes. Gendelman et al. (14) and Schuitemaker et al. (28) isolated blood monocytes by countercurrent centrifugal elutriation and cultured the cells in the presence of macrophage colony-stimulating factor, a cytokine that induces maturation of monocytes into macrophages. In contrast, we isolated the cells by adherence to plastic, and the differentiation process of the monocytes took place in the presence of autologous lymphocytes and human serum over 5 days. Several investigators have shown that the susceptibility of macrophages to HIV infection is greatly influenced by their stage of differentiation (21, 26, 33). It is of special interest that Schrier et al. (26) showed that efficient virus expression in monocyte cultures from HIV-1-infected individuals is dependent on the presence of lymphocytes during the first 24 h of culture. Similarly, Ibanez et al. (16) found that productive infection of MDM with human cytomegalovirus is dependent on in vitro maturation of the blood monocytes in the presence of stimulated lymphocytes. Thus, in view of the importance of the stage of differentiation of monocytes/macrophages, it is not surprising that different isolation and culture procedures may give seemingly contradictory results on the susceptibility of monocytes/macrophages to HIV infection.

Mononuclear phagocytes have been implicated as the primary target for HIV infection and may also be of importance as a site for persistence and dissemination of the infection (13). Furthermore, authors who have reported that only non-syncytium-inducing HIV-1 variants are capable of infecting monocytes/macrophages have suggested that these variants have an essential role during transmission and persistence of HIV-1 (28, 36). However, the present study reemphasizes that such speculations may be premature. It is clear that the ability of individual HIV-1 isolates to replicate in monocytes/macrophages is dependent on how the cells are cultured, but at present it is not clear which culture technique is more representative of the in vivo situation. However, it is likely that a significant proportion of the monocytic phagocytes in an infected individual will be susceptible to infection by rapid/high, syncytium-inducing HIV-1 variants at any given time. In accordance with this, we have found that rapid/high, syncytium-inducing HIV-1 variants can be transmitted both sexually and from mother to child (10, 25).

In conclusion, we have demonstrated that HIV-1 isolates can infect both mononuclear phagocytes and lymphocytes, irrespective of the biological phenotype and passage history of the isolate. The differences between these results and certain previously published studies are likely to be due to technical differences in how the monocytes/macrophages were isolated

and cultured. It remains to be determined which culture procedure is more relevant for the *in vivo* situation.

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